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<p>(54) Title: LOCALIZATION AND PROPAGATION OF NEURAL AND NEURONAL PROGENITOR CELLS</p>		
<p>(57) Abstract</p> <p>The present invention provides neuronal progenitor cells which have been identified in histological sections of the adult human brain. The present invention also provides methods to localize, characterize, harvest, and propagate neuronal progenitor cells derived from human brain tissue. Additional methods are provided for introducing and expressing genes in the brain.</p>		

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LOCALIZATION AND PROPAGATION OF NEURAL AND NEURONAL PROGENITOR CELLS

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10 The present application claims the benefit of U.S. Provisional Patent Application Serial No. 60/079,226 filed March 25, 1998.

FIELD OF THE INVENTION

15 The present invention relates to neuronal progenitor cells which have been identified in both tissue cultures and histological sections of the adult human brain. The present invention provides methods for the localization, characterization, harvest, and propagation of neuronal progenitor cells derived from adult humans.

BACKGROUND OF THE INVENTION

20

 The damaged adult mammalian brain is incapable of significant structural self-repair. Terminally differentiated neurons are incapable of mitosis, and compensatory neuronal production has not been observed in any mammalian models of structural brain damage (Korr, 1980; Sturrock, 1982). Although varying degrees of
25 recovery from injury are possible, this is largely because of synaptic and functional plasticity rather than the frank regeneration of neural tissues. The lack of structural plasticity of the adult brain is partly because of its inability to generate new neurons, a limitation that has severely hindered the development of therapies for neurological injury or degeneration. Indeed, the inability to replace or regenerate damaged or dead
30 cells continues to plague neuroscientists, neurologists, and neurosurgeons who are interested in treating the injured brain. During the last several years, however, a considerable body of evidence has evolved that suggests a marked degree of cellular plasticity in the adult as well as in the developing CNS. In particular, recent work on

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neural progenitor cells, derived from both embryos and adults, has suggested strategies for directed neuronal regeneration and structural brain repair. These include the use of neural stem cells which are the multipotential progenitors of neurons and glia that are capable of self-renewal (Davis, 1994; Gritti, 1996; Kilpatrick, 1993; 5 Morshead, 1994; Stemple, 1992; Goldman, 1996; Weiss, 1996a).

In the adult human brain, both neuronal and oligodendroglial precursors have been identified as well, and methods for their harvest and enrichment have been established. Neural precursors have several characteristics that make them ideal vectors for brain repair. They may be expanded in tissue culture, providing a 10 renewable supply of material for transplantation. Moreover, progenitors are ideal for genetic manipulation and may be engineered to express exogenous genes for neurotransmitters, neurotrophic factors, and metabolic enzymes (reviewed in Goldman 1998; Pincus 1998; and Goldman & Luskin 1998).

In embryonic neurogenesis, the proliferation of neuronal precursors 15 takes place at the surface of the central canal lining the neural tube (Jacobson, 1991). The central canal ultimately forms the ventricular system of the adult. This neurogenic layer is referred to as the ventricular/subventricular zone in development, and the ependymal/subependymal zone (SZ) in adults (Boulder Committee, (1970). In development, mitogenesis in the ventricular/subventricular zone is followed by the 20 migration of newly generated neurons and glia along radial guide fibers into the brain parenchyma, including that of the cortical plate (LaVail, 1971; Rakic, 1971; Rakic, 1974; Sidman, 1973).

A variety of signals, including both humoral and contact-mediated factors, have been described which influence the proliferation, differentiation, and 25 survival of stem cells and their progeny. Work on model systems derived from the peripheral nervous system has suggested that the neurotrophins (Anderson, 1986; DiCicco-Bloom, 1993; Murphy, 1991; Sieber-Blum, 1991), neurotransmitters (Pincus, 1990), and traditional growth factors (DiCicco-Bloom, 1988; Murphy, 1994; Shah, 1994) may all influence the development of precursors *in vitro*. In the CNS, soluble 30 growth factors, particularly basic fibroblast growth factor (FGF-2) regulate neuronal precursor proliferation (DeHamer, 1994; Deloulme, 1991; Drago, 1991; Gensburger, 1987; Gritti, 1996; Kilpatrick, 1995; Kitchens, 1994; Murphy, 1990; Palmer, 1995;

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Qian, 1997; Ray, 1994; Ray, 1993; Vescovi, 1993). In mixed cell cultures derived from rat embryonic cerebrum, the addition of FGF-2 stimulated the proliferation of neuronal precursors (Gensburger, 1987). Similarly, FGF-2 stimulated the proliferation of a multipotential neural progenitor in fetal mice, which gave rise to neurons and astrocytes (Kilpatrick, 1995). Embryonic rat hippocampal, spinal cord, and olfactory neuron progenitors all have been shown to proliferate in the presence of FGF-2 (DeHamer, 1994; Deloulme, 1991; Ray, 1994; Ray, 1993). Not surprisingly, FGF-2 may also regulate precursor division in concert with other factors; this has been demonstrated in the coordinate regulation of neuronal precursor division by insulin-like growth factor I and FGF-2 (Drago, 1991). as well as oligodendrocyte precursor division by FGF-2 and platelet-derived growth factor (McKinnon, 1993; Wolswijk, 1992).

Where FGF2 had been shown to promote the division of neuronal precursor cells and, hence, the specific generation of neurons, epidermal growth factor (EGF) has also been shown to influence the proliferation of uncommitted neural precursors (Kitchens, 1994; Lu, 1996; Ray, 1994; Reynolds, 1992b; Reynolds, 1992a; Santa-Olalla, 1995; Weiss, 1996b). In dissociated cultures of embryonic mouse striata grown in suspension without culture substrata, EGF induced the proliferation of progenitor cells and the formation of floating "neurospheres" of cells, which expressed nestin (Reynolds, 1992a). Nestin is an intermediate filament protein expressed not only by CNS stem cells (Dahlstrand, 1992; Lendahl, 1990a), but also by young neurons reactive astrocytes, and radial glia. When these neurospheres were dissociated and plated onto poly-L-ornithine-coated plates, γ -aminobutyric acid- and substance P-expressing neurons and glial fibrillary acidic protein-expressing astrocytes were generated (Ahmed, 1995). Similar effects were reported in adult striatal cultures (Reynolds, 1992b). In this culture preparation, the actions of EGF were mimicked by its membrane-bound homolog, transforming growth factor α , but not by nerve growth factor, FGF-2, platelet-derived growth factor, or transforming growth factor β . A similar action of EGF on precursor cells derived from embryonic and adult rat spinal cord has also been reported (Ray, 1994; Weiss, 1996).

Although it is now possible to isolate and cultivate populations of neural precursors *in vitro*, the ability to direct specific neuronal phenotypes has

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remained elusive. In the EGF-generated sphere model, multipotent progenitors differentiated into neurons, which expressed γ -aminobutyric acid and substance P, as well as astrocytes and oligodendrocytes (Reynolds, 1992b; Reynolds, 1992a; Vescovi, 1993; Weiss, 1996b). Other neuronal phenotypes were rare, and their directed
5 differentiation into defined transmitter phenotypes has not yet been demonstrated. In this regard, Raff et al. (Raff, 1988; Raff, 1983) suggested that growth factors control the development of a bipotential glial progenitor. Sequential exposure to specific combinations of platelet-derived growth factor, ciliary neurotrophic factor, and neurotrophin 3 can direct clonal expansion of the oligodendrocyte/Type 2 astrocyte
10 (O2A) progenitor cell *in vitro*, and drive an intrinsic clock that times oligodendrocyte development (Barres, 1994; Lillien, 1988; Raff, 1988; Temple, 1985). Nonetheless, a similarly directed differentiation of multipotent stem cells along specific neuronal lines has not yet been clearly demonstrated.

The persistence of neuronal precursors in the adult mammalian brain
15 may permit the design of novel and effective strategies for central nervous system repair. However, although methods for the characterization and propagation of progenitors derived from adult rodents have been described, no such methods have allowed the high-yield harvest of purified native progenitors. Furthermore, no methods have been reported for obtaining or propagating such progenitor cells from
20 adult human brain tissue.

SUMMARY OF THE INVENTION

The present invention provides human neural or neuronal progenitor
25 cells isolated and enriched from non-embryonal brain tissue of a human.

Another aspect of the present invention is a method of propagating neurons from progenitor cells derived from brain tissue by serially applying FGF2 and BDNF to the cells.

The present invention also provides a method of treating neurological
30 damage by transplanting or implanting neuronal progenitor cells into the brain of a human patient. Human neuronal progenitor cells isolated from an adult human using

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the methods of the present invention are transplanted or implanted into the brain of a patient.

Yet another aspect of the invention is a method of enhancing the survival and function of neural or neuronal precursor cells or the cells descended from the neural or neuronal precursor cells by transducing the neural or neuronal precursor cells with a gene encoding an autocrine neurotrophin or an adhesion molecule.

A further embodiment of the invention is a method of treating a patient with a neurological disease resulting from the loss of expression or mutation of a gene required for neuronal function. A gene which encodes a functional protein which complements the loss of expression or mutation of the gene required for neuronal function is transfected into postnatal or adult human neuronal progenitor cells. The neuronal progenitor cells are then introduced into the brain of the patient.

The present invention also provides a method of detecting neural or neuronal progenitor cells. An antibody, which is directed against an RNA binding protein that is selectively and specifically expressed by neural or neuronal progenitor cells when compared to other cell types, is contacted with cells and those cells which are bound by the antibody are detected.

The present invention also relates to a method of separating human, non-embryonal neural or neuronal progenitor cells from a mixed population of cells from human brain tissue. In accordance with this method, a promoter which functions only in the human postnatal neural and neuronal progenitor cells is selected. A nucleic acid molecule encoding a fluorescent protein, under control of said promoter, is then introduced into the mixed population of cells. The non-embryonal neural or neuronal progenitor cells thereof are allowed to express the fluorescent protein. The fluorescent cells are separated from the mixed population of cells, where the separated cells are the neural or neuronal progenitor cells.

Yet another embodiment of the invention is a method of expressing a gene in the brain of a patient. Adult human neuronal progenitor cells are isolated. The cells are transformed with a gene and are then transplanted into the brain of a patient. The gene is then expressed in the brain of the patient.

Prior to this invention, a method for progenitor cells were only available from embryos. However, this source is problematic due to legal and ethical

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concerns resulting from the harvesting of embryonic tissue. Furthermore, the embryonic tissue may not be immunologically compatible with the patient's tissue. The present invention provides a method for isolating and propagating progenitor cells isolated from the brain tissue of an adult human. The isolated progenitors can then be propagated *in vitro* or *in vivo* to treat nervous system damage. This method provides a non-embryonic source of progenitor cells, that may be derived from the patient's own tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

10

Figure 1 shows that cultures of the adult rat SZ typically display a burst of neuronal outgrowth during the first 11 days *in vitro* (*DIV*) but suffer virtually complete neuronal loss during the 2 weeks thereafter. However, in the presence of BDNF (20 ng/ml), new adult neurons survived substantially longer than did their counterparts grown in unsupplemented media. Figure 1A shows adult rat SZ neurons grown in control medium after 22 days. Figures 1B and 1C show sister cultures grown in BDNF after 36 and 57 days *in vitro*, respectively.

Figure 2 shows a region sampled for culture and immunohistology. Figures 2A-B are pre- and post-operative coronal MRI images of a 37 year-old female with refractory epilepsy and radiographic mesial temporal sclerosis. Figure 2A shows atrophic sclerosis of the right hippocampus and dentate gyrus (*arrow*). Figure 2B is of the same patient, 2 weeks after surgical resection. Figure 2C shows a schematic coronal view of temporal lobe, showing the subventricular region typically sampled for culture. Cortical specimens were typically obtained from the adjacent inferior temporal gyrus (modified with permission from Kirschenbaum et al., 1994 (Kirschenbaum, 1994, which is hereby incorporated by reference)).

Figure 3 shows that human temporal SZ-derived neurons are physiologically active and are the product of neurogenesis *in vitro*. A-F, cultures were challenged with high K^+ to seek evidence of neuron-like depolarization-induced increments in cytosolic calcium (Ca^{2+}_i). In this plate, a temporal SZ culture was tested after 28 days *in vitro*, after loading with the Ca^{2+} -sensitive dye fluo-3. Figure 3A is a phase micrograph of two adjacent cells, one neuron-like and the other

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astrocytic. Scale bar = 25 μ m. Figure 3B shows their baseline levels of Ca^{2+}_i , as viewed by confocal microscopy with laser scanning at 488 nm. Figure 3C depicts the same two cells within seconds after exposure to 60 mmol/ K^+ . The neuron-like cell increased its Ca^{2+}_i rapidly and reversibly, in contrast to the cocultured astrocytes.

- 5 Figure 3D shows that with the addition of tetrodotoxin (TTX) (1 μ mol/L; Sigma Chemical Co., St. Louis, MO), K^+ -stimulation yielded a more than sixfold rise in neuronal cytosolic Ca^{2+}_i , whereas astrocytic Ca^{2+}_i increased less than twofold. The depolarization-induced Ca^{2+} increment of this cell suggested its neuronal phenotype, as did the TTX accentuation of its Ca^{2+}_i response. The increased density of TTX-
- 10 sensitive Na^+ channels in neurons, relative to glia, would have been expected to yield a neuron-selective enhancement of the K^+ -stimulated Ca^{2+}_i response by TTX. Figure 3E, at withdrawal of K^+ from the medium, each cell returned to its resting Ca^{2+}_i level. Figure 3F, after addition of the calcium ionophore lasalocid (50 mmol/L, Sigma), added as a positive control to maximize Ca^{2+} entry in both cells. These results
- 15 suggested the activity of voltage-gated calcium channels in the adult-derived neurons. Figures 3G and 3H show cultures which were stained for neuron-specific antigens and were subjected to ^3H -thymidine autoradiography. Figure 3G depicts a MAP-5 $^+$ cell observed in dissociate culture after 15 days *in vitro*. Figure 3H shows a cell which has incorporated ^3H -thymidine *in vitro*, suggesting its origin from precursor mitosis
- 20 (Kirschenbaum, 1994, which is hereby incorporated by reference).

- Figure 4 demonstrates that the adult human subependyma harbors scattered islands of apparent progenitor cells. The ependyma/subependyma lining the temporal horn of the lateral ventricle, deep to the inferior temporal gyrus, in a 27
- 25 year-old man with mesial temporal sclerosis is shown. This region had distinct squamous ependymal and cuboidal subependymal layers, each 1-2 cells deep. Figures 4A-B show scattered islands of subependymal cells expressed the neural progenitor cell marker musashi. These sections were immunoperoxidase stained for musashi using anti-mouse musashi IgG. Figures 4C-D show loose aggregates of adult SZ cells also expressed Hu proteins, a triad of early, neuron-specific RNA-binding
- 30 proteins recognized by MAb 16A11 (Baram, et al., 1995; Szabo, et al. 1991, which are hereby incorporated by reference). Immunostaining revealed frequent pockets of Hu^+ scattered about the ventricular epithelium (Scale = 50 μ m.).

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Figure 5 demonstrates that serial treatment with fibroblast growth factor-2 ("FGF2") and brain-derived neurotrophic factor ("BDNF") allows for the expansion and survival of neurons arising from human subependymal zone ("SZ"). Figure 5A shows cell outgrowth from adult human SZ, 9 wks *in vitro*. The explant was exposed to FGF2 for a week, in the presence of ^3H -thymidine, then to BDNF for 2 months, then fixed and stained for the neuronal marker MAP-2. Figure 5B shows that two of these MAP-2⁺ neurons incorporated ^3H -thymidine during their first week *in vitro* (aggregations of silver grains denoted by *arrows*), indicating mitotic neurogenesis during the period of FGF2 exposure. Figure 5C is a high power visualization of the cell *asterisked* in Figures 5A-B, stained for MAP-2. Figure 5D shows the cells after ^3H -thymidine autoradiography. Figure 5E is another outgrowth from an adult temporal SZ explant, 7 weeks in culture. This sample was also raised in FGF2 followed by BDNF, then subjected to confocal imaging of the fluorescence signal emitted by the calcium indicator dye fluo-3; this was done to assess neuronal responses to depolarizing stimuli. Figure 5F depicts the baseline fluo-3 fluorescence signal from the neurons indicated in phase in 5E (*arrows*). Figure 5G was taken immediately after depolarization by 60 mM KCl. A similar response to 10 μM glutamate was observed. In each of these photos, the Scale = 25 μm .

Figure 6 shows that rare neocortical explants also exhibited neuronal production and outgrowth. In cultures derived from 2 patients, neuronal outgrowth was noted from explants derived from both the SZ *and* cortex. This suggested that temporal cortex might harbor residual neuronal precursors, analogous to those of the SZ. Figure 6A shows a field of neurons found in a temporal neocortical-explant culture after 9 wks *in vitro*, revealing a congregation of neurons lying upon a field of ependymal cells and glia. This culture was derived from a 28 year-old man, and was treated sequentially with FGF-2 (1 wk) and BDNF (8 wks). The asterisk in Figure 6A corresponds to the same position as that in Figures 6B-E. Figure 6B is a higher magnification within Figure 6A (note: Figures 6B-E are rotated 90° counter-clockwise with respect to Figure 6A). Figure 6C demonstrates that immunostaining for MAP-2 indicates the neuronal identity of these cells. Figure 6D provides the baseline calcium signal of these neurons which was determined by confocal imaging their emission to argon laser excitation at 488 nm, after loading the cells with fluo-3. Figure 6E shows

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the marked increase in fluo-3 fluorescence typical of the neuronal calcium response to K^+ -depolarization. Figures 6F-G shows that of 11 MAP-2⁺ cells in Figure 6B, four were found to have incorporated ³H-thymidine⁺; two of these are shown after ³H-thymidine autoradiography in Figure 6F (*arrows*, in phase), and after staining for
5 MAP-2 in Figure 6G. The remaining seven neurons in Figure 6B may have been cells that committed to neuronal differentiation without intervening cell division.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention provides neuronal progenitor cells isolated from the non-embryonal tissue of the human brain. This novel population of cells can be isolated from the ependymal layer or the subependymal layer of the adult human brain. A preferred embodiment of the present invention is where the cells are isolated from the temporal subependyma.

15 To identify neuronal precursor cells which persist in the adult human brain, methods were developed to culture SZ explants derived from adult human temporal lobes, removed for the treatment of intractable epilepsy (Kirschenbaum, 1994 and Pincus 1998, which are hereby incorporated by reference). Specimens were divided into cortical, subcortical, and periventricular zone-derived groups and were
20 explanted into tissue cultures using techniques previously established for the adult canary and rat brain (Goldman, 1990; Goldman, 1992a; Kirschenbaum 1995, which are hereby incorporated by reference). Both explants and monolayer cultures of the SZ yielded neurons, whose identity was confirmed by immunolocalization of a panel of neuron-selective antigens. In rare cases, new neurons were also generated from
25 neocortical tissue. Furthermore, temporal SZ dissociates exposed to ³H-thymidine in culture yielded ³H-thymidine-labeled, microtubule-associated protein 2-expressing cells, indicative of neurons newly generated *in vitro* (Figure 2). Of note, the neurons generated by these precursors assume neuronal function as well as antigenicity. Confocal calcium imaging of neurons migrating from adult human SZ explants
30 showed that they responded to both potassium depolarization and glutamate with sharp increases in cytosolic calcium, indicating their development of mature neuronal response characteristics (Figure 3).

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A preferred type of human progenitor cells express an RNA-binding protein which characterizes early neural or neuronal phenotype. More particularly, to identify neural progenitor cells and their daughters *in vivo*, brain samples were immunostained for RNA-binding proteins that characterize early neural or neuronal phenotypes, the musashi and Hu proteins, respectively. In a preferred embodiment of the invention the RNA-binding protein that characterizes the early uncommitted neural phenotype is musashi. Musashi, a 39 kD RNA-binding protein initially described in *Drosophila* and *Xenopus*, is expressed in development by mitotic neural progenitors, including CNS stem cells. In the adult rat brain, musashi expression is limited to progenitor cells of the lateral ventricular and olfactory subependyma. It is not expressed by mature neurons or oligodendrocytes, but is expressed at a low level by parenchymal astrocytes. In an alternative embodiment of the invention, the RNA-binding protein that characterizes the early neuronal phenotype is a Hu protein. In contrast to Musashi, Hu comprises a family of 35-42 kD proteins, at least three of which, HuD, HuC, and HelN1, are expressed selectively by neurons and their committed post-mitotic progenitors. HuD, HuC, and HelN1 are preferred embodiments of the Hu protein. In the CNS, Hu appears very early in neuronal ontogeny, but is not expressed by cycling progenitors; it is thus an effective marker for neuronal daughter cells still within the SZ (Baram, et al., 1995, which is hereby incorporated by reference).

In preferred embodiments, cells are isolated from patients with epilepsy, requiring brain tissue resection for treatment of trauma, cerebral edema, or aneurysmal repair.

The present invention also relates to a method of separating human, non-embryonal neural or neuronal progenitor cells from a mixed population of cells from human brain tissue. In accordance with this method, a promoter which functions only in the human non-embryonal neural and neuronal progenitor cells is selected. A nucleic acid molecule encoding a fluorescent protein, under control of said promoter, is then introduced into the mixed population of cells. The non-embryonal neural or neuronal progenitor cells thereof are allowed to express the fluorescent protein. The fluorescent cells are separated from the mixed population of cells, where the separated cells are the non-embryonal neural or neuronal progenitor cells. This procedure is

more fully described in U.S. Patent Application Serial No. 08/787,788, which is hereby incorporated by reference.

Illustrations of possible cell and promoter combinations which can be used in accordance with the present invention include: a neuronal progenitor and an NCAM promoter (Holst et al. 1994, which is hereby incorporated by reference); a neuronal progenitor and an α 1-tubulin promoter (Gloster et al. 1994, which is hereby incorporated by reference); neuronal or neural progenitors and the musashi protein promoter (Nakamura 1994; Richter 1990; Sakakibara 1996; and Sakakibara 1997, which are hereby incorporated by reference); and neuronal or neural progenitors and a Hu protein promoter (Szabo 1991, which is hereby incorporated by reference).

This approach was first established as a means of separating neuronal progenitors from a mixed cell population, using the early neuronal T α 1 tubulin promoter (Wang. 1998, which is hereby incorporated by reference). However, this technique is equally amenable to use with other promoters, such as the tyrosine hydroxylase promoter activated in dopaminergic neurons. As such, this technique may facilitate the selection of transmitter-defined neurons, such as dopaminergic cells, for implantation.

The present invention provides a method of propagating neurons from human progenitor cells isolated from the brain, by serially applying fibroblast growth factor-2 ("FGF-2") and brain-derived neurotrophic factor ("BDNF") to the cells. FGF-2 and BDNF support progenitor division and neuronal maturation and viability, respectively. The effect of both are enhanced by simultaneous treatment with insulin-like growth factor-1 (i.e. IGI-1), which is added to tissue cultures together with FGF2 and BDNF.

In one embodiment, the cells are propagated in culture. In a preferred embodiment, the progenitor cells being propagated and differentiated are derived from fetal brain tissue. Alternatively, the brain cells being propagated and differentiated are derived from juvenile or adult brain tissue.

The method of the present invention may also be used to stimulate neuronal precursors and induce the formation of neurons *in situ*, for example with FGF2 and/or BDNF intraventricular injection (Kirschenbaum 1995; Pincus et al. 1998; and Zigova et al. 1998, which are hereby incorporated by reference). For *in situ*

treatment, the brain tissue may be that containing from endogenous subependymal or cortical progenitors. Various benefits of *in situ* treatment are discussed more fully below.

5 The persistence of such neuronal precursors in the adult brain as well as the isolation of stem cells lines may be exploited clinically in several ways. The most obvious strategy for brain repair is transplantation. The present invention provides a method for treating neurological damage by transplanting or implanting neuronal progenitor cells into a human brain.

10 In a preferred embodiment of the invention, the neuronal progenitor cells are transplanted or implanted into the subendyma of the neonatal or juvenile human ventricular wall, for the treatment of perinatal brain injury, germinal matrix hemorrhage, or cerebral palsy.

In another preferred embodiment of the invention, the method of transplanting or implanting neuronal progenitor cells into the brain is used to treat 15 Parkinson's Disease. Transplantation has been used in the treatment of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced and idiopathic Parkinson's Disease by implantation of fetal mesencephalon (Freed, 1993; Spencer, 1992; Widner, 1992, which are hereby incorporated by reference). However, the difficulties with the use of fetal material are many fold; tissue availability remains a problem, and political and 20 ethical issues continue to hamper progress in this field. Considering these problems, the future of cellular replacement in the nervous system requires additional sources of transplantable material. The use of cultured neuronal cell lines would potentially obviate these difficulties (Snyder, 1997, which is hereby incorporated by reference). Whereas the best source of material for the establishment of new cell lines will likely 25 remain fetal brain, the number of fetuses necessary should be relatively few in comparison to that required for transplantation of primary material. Although the implantation of fetal mesencephalic cells had been limited by the relative scarcity of dopaminergic progenitors in the grafts, the approaches to separating specific transmitter phenotypes may allow the enrichment of desired cell types described 30 above. These techniques are based on fluorescence-activated cell sorting of cells transfected with fluorescent transgenes placed under the control of cell-specific promoters (Wang, 1998, which is hereby incorporated by reference).

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In another embodiment of the invention, transplantation or implantation of adult neural progenitors may be used to treat a demyelinating disease. Transplantation or implantation of isolated and/or expanded human progenitors may also be used to treat ischemic brain damage, stroke, or traumatic brain injury.

5 As an alternate strategy, the presence of precursors in adult brain suggests a potential autologous source for the generation of cultured lines, which may then be reimplanted into the donor (Gage, 1995; Suhonen, 1996, which are hereby incorporated by reference).

10 Another embodiment is that cultured adult neuronal or neural progenitors may be immortalized by transduction or transfection with immortalizing oncogenes such as v-myc or the large T antigen of SV40 virus (e.g. Flax et al., 1998, which is hereby incorporated by reference, for use with fetal progenitors).

15 Another approaches may also be used for the possible treatment of demyelinating disease. Oligodendrocyte Type 2 astrocyte progenitors have been injected into demyelinating lesions in adult rat (Franklin, 1996; Gensert, 1997; Groves, 1993, which are hereby incorporated by reference) or canine (Archer, 1997, which is hereby incorporated by reference) spinal cord and are capable of producing extensive remyelination. Further, in addition to neuronal precursor pools, analogous progenitors for oligodendrocytes persist in the mature human brain (Armstrong, 1992; 20 Kirschenbaum, 1994; Scolding, 1995, which are hereby incorporated by reference).

The isolated neuronal progenitor cells may be transformed with a number of therapeutic genes.

25 In a preferred embodiment, the gene encodes an autocrine neurotrophin. Preferred autocrine neurotrophins include FGF2, BDNF, and IGF1. The invention also provides neuronal precursor cells transformed with a gene encoding an autocrine neurotrophin.

30 In another preferred embodiment, the gene encodes an adhesion molecule. Preferred adhesion molecules include N-CAM, NgCAM, other members of the immunoglobulin family of adhesion molecules, a member of the integrin-family of proteins, or a member of the connexin family of proteins. The invention provides neuronal precursor cells transformed with a gene encoding an adhesion molecule.

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The nucleic acid molecule encoding a gene which is to be expressed can be inserted into a suitable host cell. Various methods for transforming host cells are known in the art. Preferred methods include microinjection, the DEAE-dextran method, electroporation, diffusion, or the use of viral vectors. Preferred viral vectors include retroviruses, adenoviruses, adenoassociated viruses, herpesviruses, and lentiviruses.

The adult human-derived progenitor cells of the present application may also be used as vectors for cell-based gene therapy (Lacorazza and Snyder, which are hereby incorporated by reference). In addition to strategies directed at replacing a particular cell type lost to neurodegenerative disease, progenitor cells may also serve as a vector for gene therapy. The present invention provides a method of expressing a gene in the brain of a patient. Human neuronal progenitor cells are isolated from an adult human and transformed with the gene of interest. The transformed progenitor cells are then transplanted into the brain of the patient.

The present invention also provides a method of enhancing the survival and function of neural or neuronal precursor cells or cells descending from the neural or neuronal precursor cells. This enhancement is carried out by transducing the neural or neuronal precursor cells with a gene encoding an autocrine neurotrophin or an adhesion molecule.

Yet another embodiment of the invention provides a method of detecting neural or neuronal progenitor cells. An antibody directed against a protein, which is preferentially associated with neural or neuronal progenitor cells when compared to other cell types, is contacted with cells. The cells which bind to the antibody are then detected.

Another strategy allows the selective harvest of these cells. Enzymatic dissociation of the adult human ventricular lining and/or brain parenchyma is followed by transfection with plasmid DNA containing a progenitor specific promoter sequence placed 5' to reporter genes (e.g., lacZ or GFP). Progenitors, defined by the transcriptional activation of the introduced promoter sequences and consequent expression of the fluorescent reporter gene), can then be identified and viably selected via FACS.

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The present invention further provides a method of expressing a gene in the brain of a patient. Isolated adult human neuronal progenitor cells are transformed with the gene. The transformed cells are transformed into the brain of a patient. Once implanted, the gene is expressed in the brain of the patient.

5

EXAMPLES

Example 1 - Materials and Methods

10 *Tissue samples*

Adult human temporal lobe was obtained during anterior temporal lobectomy, performed for the treatment of medically-refractory epilepsy (Figure 4). The study was limited to patients not known to harbor tumor of any origin, and
15 excluded patients with either radiographically or pathologically-identified dysplasias. The sample included 16 patients, ranging from 6 months to 55 years of age (9 males and 7 females). Approval was obtained from both the New York Hospital-Cornell and Columbia-Presbyterian Institutional Review Boards. Pre-operative MRI was performed on all patients, among whom the most common finding was medial
20 temporal sclerosis (Figure 4), although several were radiographically normal. Three patients with radiographic cortical dysplasias were excluded from the study. Routine post-operative pathologic examination of the cortex and hippocampus, with hematoxylin and eosin staining of 10 μ m paraffin sections, typically revealed neuronal drop-out and marginal sclerosis, consistent with temporal sclerosis. No
25 occult glioma or other neoplasia was found in these samples.

Culture preparation

Resections were dissected into neocortical and periventricular samples,
30 the latter including both the ependymal and subependymal layers (jointly denoted as subependymal/ ependymal zone, SZ). Explant cultures were prepared as described (Kirschenbaum, 1994, which is hereby incorporated by reference). In brief, tissue samples was cut into roughly 0.3 mm³ explants, that were plated on murine laminin in 35 mm petri dishes (Falcon Primaria), and cultured at 37°C in 5% CO₂/95% air.

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Media

Cultures were grown in Dulbecco's modified Eagle's medium/Ham's F-12, supplemented with N2 (Bottenstein, 1979, which is hereby incorporated by reference), 15 mM HEPES, 8.5 mg/ml glucose, 6.5 mM L-glutamine, 1.5 mM sodium pyruvate, 30 ng/ml tri-iodothyronine, 1.35 mg/ml bovine serum albumin, 10 mM non-essential amino acids, 40 U/ml penicillin/streptomycin, and 5% fetal bovine serum (Gibco). For the first week *in vitro*, explants were grown in this base medium, with or without added FGF2 (20 ng/ml); all cultures were exposed to ³H-thymidine (0.25 μCi/ml) during the first week, but not thereafter. The cultures were then switched to media containing 10% FBS at 8 DIV, half with and half without added BDNF (20 ng/ml; Regeneron). All plates received half-volume changes with their test media twice weekly.

Immunocytochemistry

In vitro. Cultures were incubated for up to 9 weeks, fixed with 4% paraformaldehyde, and immunostained for the neuron-selective protein MAP-2 (microtubule associated protein-2;) (Bernhardt, 1984, which is hereby incorporated by reference). Two different rabbit anti-MAP-2 antisera were used (courtesy of Drs. S. Halpain and I. Fischer), each at 1:1000, followed by biotinylated anti-rabbit IgG (1:100-1:200) and Texas Red-conjugated avidin (Vector). Neurons were defined as those cells with typical multipolar morphology and immunoreactivity for MAP-2.

Surgical samples. Both neuronally-committed and uncommitted subependymal progenitor cells were identified in 5 resected temporal lobe samples, using the cell type-selective markers Hu for neurons, and musashi for uncommitted progenitors (see below). The samples were each taken at surgery, and immersed immediately in 4% paraformaldehyde. From each, 12 μm cryostat sections of the forebrain were cut and mounted onto Vectabond-subbed slides. To identify Hu-immunoreactive SZ cells, MAb 16A11, which recognizes all 3 neuronal members of the Hu family (Marusich, 1992; Marusich, 1994, which are hereby incorporated by reference), was used as previously described, at 12.5 μg/ml, with avidin-biotin amplification, peroxidase-conjugated avidin detection, and diaminobenzidine (DAB)/H₂O₂ development (Barami, 1995, which is hereby incorporated by reference).

Musashi was localized using rat monoclonal anti-mouse musashi IgG (Sakakibara, 1997; Sakakibara, 1996, which are hereby incorporated by reference), 1:500 overnight at 4°C, followed by biotinylated anti-rat IgG (1:100), and peroxidase-conjugated avidin-biotin (Vector), with DAB/H₂O₂ development.

5

³H-thymidine labeling and autoradiography

The uptake of ³H-thymidine (0.2 µCi/plate, from 1 Ci/ml stock; 5 Ci/mM, Amersham) by antigenically-defined neurons was used as an index of antecedent precursor cell S-phase and division *in vitro*. Cultures were exposed to ³H-thymidine during their first 7 days *in vitro*, after which a complete media-exchange removed residual isotope. After immunocytochemistry, the plates were air-dried, then autoradiographed as described (Kirschenbaum, 1994, which is hereby incorporated by reference). Briefly, Kodak NTB-3 emulsion was added to each petri dish for 3 min, then withdrawn by Pasteur pipette, leaving a remnant gel. After a week at 4°C in the dark, the plates were developed with Kodak D-19, fixed, washed, covered with 50% glycerol in 0.1M phosphate buffer, and observed with an Olympus IX70 microscope.

20

Calcium imaging

To identify neurons physiologically, cells in selected plates were challenged with a depolarizing stimulus of 60 mM K⁺, during which their cytosolic calcium levels were observed. Calcium imaging was performed using confocal microscopy of cultures loaded with fluo-3 acetomethoxyester (fluo-3, Molecular Probes), as described previously (Kirschenbaum, 1994; Goldman, 1996a, which are hereby incorporated by reference). A Bio-Rad MRC600 confocal scanning microscope, equipped with an argon laser and coupled to a Nikon Diaphot 300 microscope, was used to image the fluo-3 signal. Each experiment was carried out at 25°C in HBSS, with 60 mM K⁺ exchanged for 60 mM Na⁺ in the depolarizing solution.

30

These neurons were previously reported to display a mean calcium rise of >400% to 60 mM K⁺ *in vitro*; this contrasted to an astrocytic calcium response of <20%, and undetectable oligodendroglial responses (Kirschenbaum, 1994, which is

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hereby incorporated by reference). On this basis, a 2-fold calcium increase to depolarization was required here for assigning neuronal identity.

Example 2 - Neuronal Outgrowth Arose from Explants of the Adult Human SZ

5 In this series, 8 of 16 brains exhibited ependymoglia outgrowth. The other 8 exhibited no cellular outgrowth of any kind, and were deemed technical failures. The likelihood of successful outgrowth appeared to depend in part upon the transit time between the operating room and culture facility; the longest latencies to
10 culture were associated with the worst explant survival and outgrowth. Among the 8 brains that exhibited outgrowth, cells with neuronal morphology and antigenicity were observed in outgrowths from 4 (a 6 month-old female, 27 year-old male, and 35 and 52 years old females). These cells were typically found upon a field of
15 ependymal cells and astrocytes, as ovoid cell bodies with thin processes (Figure 5). In 2 patients, the identity of neuron-like cells in the explant outgrowths was confirmed physiologically as well as antigenically (Figure 5).

Example 3 - Serial Exposure to FGF-2 and BDNF Promoted the Generation and Maintenance of Networks of New Neurons

20 Among cultures raised sequentially in FGF-2 and BDNF, SZ outgrowths were noted that generated morphologically complex neuronal networks, some of which survived >2 months in culture (Figure 5). In contrast, no plates raised in the absence of BDNF maintained viable neurons beyond a month *in vitro*, and none
25 raised without *both* FGF2 and BDNF exhibited >10 neurons per explant. In selected FGF2/BDNF-treated surveillance cultures, the neuronal identity of cells was verified, both antigenically and physiologically. In one such case, 3 long-term cultures derived from one patient were subjected after 9 weeks *in vitro* to combined immunostaining for MAP-2 and autoradiography for ³H-thymidine. Among 55 MAP-2⁺ neurons
30 identified in these 3 cultures, 10 were double labeled as ³H-thymidine⁺/MAP-2⁺ (Figures 2 and 3). These data indicate division of mitotic progenitors during the first week *in vitro*, during the period of concurrent FGF-2 and ³H-thymidine exposure, though they do not necessarily indicate a direct causal relationship between FGF and neuronal mitogenesis.

Example 4 - Neurons Generated from Adult SZ Explants in the Presence of FGF2/BDNF Developed Neuronal Calcium Responses to Depolarization

5 To confirm the ability of these cells to respond in a neuronal fashion to depolarizing stimuli, selected cultures (n = 6, derived from 2 brains) were loaded with the calcium indicator dye fluo-3, and exposed to 60 mM K⁺ during confocal microscopy. Glial responses to depolarization were minimal under these culture
10 conditions, as previously noted (Kirschenbaum, 1994, which is hereby incorporated by reference). In contrast, neuron-like cells verified as such displayed rapid, reversible, >100% elevations in cytosolic calcium in response to K⁺, consistent with the activity of neuronal voltage-gated calcium channels (Figure 5). The neuronal phenotype of these cells was then validated antigenically, by immunostaining for
15 MAP-2 (Figure 5).

Example 5 - Neuronal Outgrowth was Noted on Rare Occasion from Neocortical Explants

20 In cultures derived from two patients (a 27 years old male and 35 years old female), neuronal outgrowth was noted in cultures derived from the neocortex as well as the SZ. These cells survived up to 9 weeks *in vitro*, expressed MAP-2, and exhibited >100% increments in cell calcium to K⁺-depolarization, consistent with their neuronal phenotype (Figure 6). Some, like their SZ-derived counterparts,
25 incorporated ³H-thymidine during the period of FGF-2 exposure. Long-term neuronal outgrowth from these cultures was limited to those that had been serially exposed to both FGF-2 and BDNF; untreated explants had no surviving neuronal outgrowth beyond a month *in vitro*. Depolarization-responsive MAP-2⁺ neurons were also identified in dissociates from a second cortical sample, derived from a 35 years old
30 female (these had not been exposed to thymidine *in vitro*).

In this series, over 800 neocortical explants were prepared from 8 different patients whose cultures exhibited at least glial outgrowth. Yet among these cortical explants, only 4, derived from 2 patients, exhibited neuronal outgrowth. Overall then, ≤0.5% of cortical explants generated any neuronal outgrowth.
35 Nonetheless, the observation of any MAP-2⁺/³H-thymidine⁺, depolarization-

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responsive neurons in these outgrowths was remarkable. in that it argued for the existence of rare neuronal progenitors in the neocortical parenchyma.

5 **Example 6 - Immunostaining Revealed Cells Expressing the Early Neural Proteins Musashi and Hu in the Subependyma**

To identify neural progenitor cells and their daughters *in vivo*, the cells were immunostained for 2 RNA-binding proteins that characterize early neuronal phenotype, the musashi and Hu proteins. Musashi, a 39 kD RNA-binding protein
10 initially described in *Drosophila* and *Xenopus* (Nakamura, 1994; Richter, 1990, which are hereby incorporated by reference), is expressed in development by mitotic neural progenitors, including CNS stem cells (Sakakibara, 1996, which is hereby incorporated by reference). In the adult rat brain, musashi expression is limited to progenitor cells of the lateral ventricular and olfactory subependyma. It is not
15 expressed by mature neurons or oligodendrocytes, but is made by parenchymal astrocytes (Sakakibara, 1997, which is hereby incorporated by reference). In contrast, Hu comprises a family of 35-42 kD proteins (Szabo, 1991, which is hereby incorporated by reference), at least 3 of which, HuD, HuC and HelN1, are expressed selectively by neurons and their committed progenitors. In the CNS, Hu appears very
20 early in neuronal ontogeny but is not expressed by cycling progenitors; in the SZ, it is an effective marker for neuronal daughter cells (Barami, 1995, which is hereby incorporated by reference).

For histological evaluation of progenitor phenotypes in the SZ, surgical samples of temporal horn ventricular epithelium were prepared as described from 5
25 patients (22 and 23 year-old males, and 9, 29, and 46 year-old females). In all 5, Hu⁺ cells were found in the temporal ventricular subependyma. These cells were generally found in small clusters; typically several multicell clusters and scattered lone cells were observed per ventricular cross-section (Figure 4). Among 18 sections of the temporal SZ, derived from 5 brain samples, an average of 10.1 ± 0.8 (mean \pm
30 SD) Hu⁺ SZ cells/mm were found; this corresponded to a density of 78 ± 22.6 Hu⁺ cells per 1000 hematoxylin-defined subependymal cells (Table 1). Within the subjacent parenchyma, all neurons and some oligodendrocytes expressed Hu-IR; no astrocytic expression of Hu was ever noted.

Table 1 - Distribution and Density of Antigenically-Defined Precursor (Musashi) and Daughter (Hu) Phenotypes in the Adult Human Temporal SZ

5

A. Musashi⁺ cells in the adult human temporal SZ (see Figure 4A)

Patient (age/sex)	Mean segment length (mm)	Total SZ cells	Musashi ⁺ cells/section	Musashi ⁺ /mm	% Musashi ⁺
29/female	11.014 ± 1.230	1123 ± 48	90 ± 9.1	8 ± 0.4	8 ± 0.6
22/male	2.473 ± 0.969	361 ± 136	26 ± 7.5	11 ± 2.1	7.4 ± 1.3
9/female	2.210 ± 0.040	411 ± 21	13 ± 2.1	6 ± 1.0	3.3 ± 0.5
OVERALL				8 ± 2.4	6.2 ± 2.6%

10

B. Hu⁺ cells in the adult human temporal SZ (see Figure 4B)

Patient (age/sex)	Mean segment length (mm) ^{1,2}	SZ cells/section	Hu ⁺ cells/section	Hu ⁺ /mm	% Hu ⁺
46/female	3.913 ± 0.703	342 ± 23	44 ± 9.1	11 ± 1.1	11.1 ± 0.9%
23/male	2.286 ± 0.456	247 ± 45	21 ± 8.1	9 ± 2.0	7.2 ± 1.6%
29/female	9.409 ± 0.227	1088 ± 36	88 ± 4.5	9 ± 0.3	8.1 ± 0.5%
22/male	1.336 ± 0.158	173 ± 13	13 ± 1.5	10 ± 0.7	7.7 ± 0.3%
9/female	1.066 ± 0.256	238 ± 38	12 ± 2.5	11 ± 0.5	4.9 ± 0.4%
OVERALL				10 ± 0.8	7.8 ± 2.2%

15 The density and distribution of musashi-immunoreactive (musashi⁺) SZ cells was similar to that of Hu⁺ SZ cells: an average of 8.4 ± 2.4 musashi⁺ cells per mm of ventricular surface were found, for a density of 62 ± 26.0 musashi⁺ cells per 1000 SZ cells (n=3 patients, 3 immunostained sections each) (Figure 4; Table 1). Since double-labeling for both proteins was infrequent in this sample, of Hu⁺ (7.8 ± 2.3%) and musashi⁺ (6.2 ± 2.6%) cells together constituted <15% of temporal subependymal zone cells.

20

Neuronal outgrowth from explants of the adult temporal SZ were previously identified, and on that basis the existence of neuronal progenitors in mature humans was inferred (Barami, 1995, which is hereby incorporated by reference). However, neuronal outgrowth from these explants, raised in high-serum

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unsupplemented media, was relatively sparse (<10 neurons/productive explant), and their survival transient. The present invention shows that these neural precursor cells could be identified *in situ*, and that their production and sustenance of new neurons could be enhanced by neurotrophins identified as such in rodents. The sequential
5 exposure of adult temporal SZ explants to FGF-2 and BDNF was associated with abundant and long-lasting neuronal outgrowth. In contrast, cultures grown in the absence of added FGF2 and BDNF exhibited neither neuronal outgrowth of this magnitude, nor survival of this duration. This was in accordance with the findings in cultures of the adult rat SZ, in which neuronal survival past the second week *in vitro*
10 required BDNF addition (Kirschenbaum, 1995, which is hereby incorporated by reference).

In adult avian and rodent SZ cultures, those neurons that emigrate from the explants comprise the newly generated pool; virtually all incorporate ³H-thymidine *in vivo*, in the days before sacrifice, or shortly thereafter in culture
15 (Goldman, 1992a; Lois, 1993; Kirschenbaum, 1995, which are hereby incorporated by reference). In adult human SZ cultures, the significant fraction of MAP-2⁺ neurons that incorporated thymidine *in vitro* suggested that many if not all of the neurons in the explant outgrowths were similarly derived from mitotic progenitors.

Neuronal progenitors in the neocortical parenchyma were exceedingly
20 rare. Nonetheless, the scarcity of these progenitors was perhaps not as surprising as was their very existence: Most previous studies have found that the source of neural precursors in both adult birds and mammals is the ventricular zone (Goldman, 1990; Lois, 1993; Morshead, 1994, which are hereby incorporated by reference), through which they are widely dispersed (Kirschenbaum, 1995; Weiss, 1996b; Nottebohm,
25 1985; Alvarez-Buylla, 1988, which are hereby incorporated by reference). Yet the neuronal precursors of the adult rodent brain have been reported to reside in parenchymal as well as ventricular sites (Richards, 1992; Palmer, 1995, which are hereby incorporated by reference), as have oligodendrocytic progenitors (Gensert, 1996, which is hereby incorporated by reference). Whether these adult cortical
30 progenitors are homologous to their counterparts in either the developing cortex (Davis, 1994, which is hereby incorporated by reference) or adult SZ is unknown.

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The nature of parenchymal neural precursor cells is unclear because of the epileptic pathology of these patients. In these samples, apparent cortical progenitors might have derived from cells in microheterotopic foci which were not apparent on routine pathologic examination. Interestingly, this opens the possibility that the heterotopic cell aggregations associated with several uncommon forms of refractory epilepsy, might include inappropriately situated neuronal precursors (Jay, 1994, which is hereby incorporated by reference). In particular, the nodular subependymal and subcortical white matter heterotopias, with their periventricular locales and granule cell predominance, might constitute reservoirs of subependymal cells with precursor potential (Dubeau, 1995; Huttenlocher, 1994; De Rosa, 1992; Raymond, 1994, which are hereby incorporated by reference). These heterotopic cell rests may be comprised of aberrant ventricular zone emigrants, that remain functionally quiescent, yet develop into neurons when presented a permissive *in vitro* environment. In this regard, it is intriguing that most patients with both nodular and laminar (band) heterotopias are female, and largely develop epilepsy during puberty (Raymond, 1994; Palmini, 1991b; Palmini, 1991a; Barkovich, 1994, which are hereby incorporated by reference). These include familial subependymal heterotopias, that appear to follow X-linked dominant inheritance (Huttenlocher, 1994, which is hereby incorporated by reference). In such cases, ectopic subependymal cells might conceivably persist as quiescent progenitors until the estrogenic stimulation associated with puberty, at which time they might assume neuronal function, with epileptogenic consequences.

In adult rodents, neurons and glial progenitors are abundant in the forebrain subependyma, in particular in the rostral dorsolateral aspect of the lateral ventricular system (Lois, 1993; Kirschenbaum, 1995; Morshead, 1994; Luskin, 1997, which are hereby incorporated by reference). While some of the neuronal progeny of these SZ cells die *in situ* (Morshead, 1992, which is hereby incorporated by reference), most appear to migrate rostrally to the olfactory bulb, in an adult manifestation of the rostral migratory stream first reported in the developing rodent brain (Altman, 1965; Frazier-Cierpial, 1989; Luskin, 1993b; Corotto, 1993; Lois, 1994, which are hereby incorporated by reference). In rodents, these new neurons migrate in chains within the subependymal milieu, without ever entering the brain

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parenchyma, in a novel form of neurophilic migration in the absence of radial guide fibers (Lois 1996; Doetsch, 1996, which are hereby incorporated by reference). In the present invention, these observations are extended to the human forebrain, where musashi and Hu expression was used to identify progenitors and their neuronally-committed daughters in the adult SZ. Significant subependymal populations of each phenotype were found. In the temporal horn of the lateral ventricle, musashi⁺ cells were scattered about, singly and apparently stochastically. Hu⁺ SZ cells were often found together in small aggregates, typically of no more than 3 cells. Whether these cells travel as chains more rostrally in the ventricular wall, and whether they are analogous to the new neurons of the rodent olfactory stream, remains to be seen.

Roughly 15% of the subependymal cells of the temporal horn expressed either musashi or Hu proteins. However, the virtual uniqueness of each sample with regards to disease state, sex, age, stereotaxic position and histological integrity made comparisons of Hu⁺ and musashi⁺ SZ cell distributions between samples difficult. It is similarly unclear whether these estimates are reflective of the distribution of musashi⁺ and Hu⁺ cells in other regions of the ventricular system: Analogy to the distribution of precursor cells in the adult rat, in which caudal regions of the ventricular system have the lowest numbers of progenitors (Kirschenbaum, 1995); Weiss, 1996b, which are hereby incorporated by reference) suggests that the area of human brain which was sampled might be expected to have a lower density of neuronal progenitor cells than more rostral regions, in particular that abutting the vestigial olfactory subependyma.

The presence of musashi and Hu⁺ subependymal cells in the adult human epileptics, and their spatial colocalization with FGF2 and BDNF-responsive neuronal precursors, argues for the presence of neural precursors in the adult human subependyma. Some progenitors might also persist within the cortex itself. Though small in numbers, the ability of these cells to yield a profusion of new neurons *in vitro* suggests the promise of generating new neurons within the adult human brain.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing

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from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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WHAT IS CLAIMED:

1. Human neural or neuronal progenitor cells isolated from non-embryonal human brain tissue.
- 5 2. The human neural or neuronal progenitor cells according to claim 1, wherein the progenitor cells are isolated from the brain's ependyma and subependyma.
- 10 3. The human neural or neuronal progenitor cells according to claim 1, wherein the cells express an RNA-binding protein which characterizes an early neural or neuronal phenotype.
- 15 4. The human neural progenitor cells according to claim 3, wherein the RNA-binding protein is a mushashi protein.
5. The human neuronal progenitor cells according to claim 3, wherein the RNA-binding protein is a Hu protein.
- 20 6. The human neural or neuronal progenitor cells according to claim 5, wherein the Hu protein is selected from the group consisting of HuD, HuC, and HelN1.
- 25 7. A method of propagating neurons from progenitor cells derived from human brain tissue comprising:
applying serially FGF2 and BDNF to the progenitor cells.
8. The method according to claim 8 further comprising:
applying IGFI to the progenitor cells.
- 30 9. The method according to claim 7, wherein the cells are propagated in culture

10. The method according to claim 7, wherein the brain tissue is fetal brain tissue.
- 5 11. The method according to claim 7, wherein the brain tissue is juvenile or adult brain tissue.
12. The method according to claim 7, wherein the cells are propagated *in situ*.
- 10 13. The method according to claim 12, wherein the brain tissue contains subependymal, subcortical, or cortical progenitors.
14. A method of treating neurological damage by transplanting or
15 implanting neuronal progenitor cells into a human brain comprising:
providing isolated adult human neuronal progenitor cells; and
transplanting or implanting said progenitor cells to the brain of
a human patient.
- 20 15. The method according to claim 14, wherein the neural or neuronal progenitor cells are transplanted or implanted into the brain's subendyma of the human neonatal or post-natal ventricular wall.
16. The method according to claim 14, wherein the neurological
25 damage resulted from a degenerative neurological disease.
17. The method according to claim 16, wherein the patient suffers from Parkinson's disease.
- 30 18. The method according to claim 16, wherein the patient suffers from a demyelinating disease.

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19. The method according to claim 14, wherein the neurological damage resulted from ischemic conditions or stroke.

20. The method according to claim 14, wherein the neurological
5 damage resulted from traumatic brain injury.

21. A method of enhancing survival and function of neural or neuronal precursor cells and cells descending from the neural or neuronal precursor cells comprising:
10 transducing neural or neuronal precursor cells with a gene encoding an autocrine neurotrophin or an adhesion molecule.

22. The method according to claim 21, wherein the gene encodes an autocrine neurotrophin which is selected from the group consisting of FGF2, BDNF, and IGF1.
15

23. A neuronal precursor cell produced by the method of claim 21.

24. The neuronal precursor cell according to claim 24, wherein the autocrine neurotrophin is selected from the group consisting of FGF2, BDNF, and IGF1.
20

25. The method according to claim 21, wherein the gene encodes an adhesion molecule.
25

26. The method according to claim 25, wherein the adhesion molecule is selected from the group consisting of N-CAM, NgCAM, and a member of the integrin-family of proteins.

27. A neuronal precursor cell provided by the method of claim 25.
30

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28. The neuronal precursor cell according to claim 27, wherein the adhesion molecule is selected from the group consisting of N-CAM, NgCAM, and a member of the integrin-family of proteins.

5 29. A method of detecting neural or neuronal progenitor cells comprising:

providing an antibody directed against a protein which is preferentially associated with neural or neuronal progenitor cells when compared to other cell types;

10 contacting cells with the antibody; and
detecting cells which bind to the antibody.

30. The method according to claim 29, wherein the protein is a Hu protein.

15

31. The method according to claim 30, wherein the Hu protein is selected from the group consisting of HuD, HuC, and HelN1

20 32. The method according to claim 29, wherein the protein is a musashi protein.

33. A method of expressing a gene in the brain of a patient, comprising:
25 cells;

providing isolated postnatal or adult human neuronal progenitor
transfecting said cells with the gene;
introducing said transfected cells to the brain of a patient; and
expressing the gene in the brain of the patient.

30 34. The method according to claim 33, wherein the patient suffers from Tay-Sachs disease.

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35. The method according to claim 33, wherein the gene encodes hexoseaminidase.

36. The method according to claim 33, wherein the patient suffers
5 from a hereditary metabolic disease.

37. The method according to claim 36, wherein the hereditary metabolic disease is mucopolysaccharidosis.

10 38. A method of separating human, non-embryonal neural or neuronal progenitor cells from a mixed population of cells from human brain tissue, said method comprising:

selecting a promoter which functions only in said human postnatal neural and neuronal progenitor cells;

15 introducing a nucleic acid molecule encoding a fluorescent protein under control of said promoter into the mixed population of cells;

allowing the non-embryonal neural or neuronal progenitor cells thereof to express the fluorescent protein; and

20 separating the fluorescent cells from the mixed population of cells, wherein said separated cells are said neural or neuronal progenitor cells.

39. A method according to claim 38, wherein said introducing is selected from the group consisting of viral mediated transformation of the mixed population of cells, electroporation, and liposomal mediated transformation of the
25 mixed population of cells.

40. A method according to claim 38, wherein said separating comprises fluorescence activated cell sorting.

30 41. A method according to claim 38, wherein the promoter is selected from a Hu promoter, a Musashi promoter, and a Nestin enhancer-promoter.

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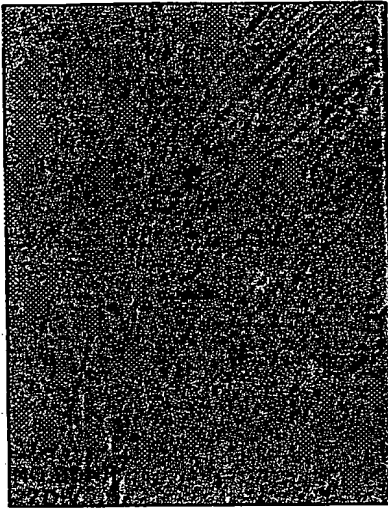


FIG. 1A



FIG. 1B



FIG. 1C

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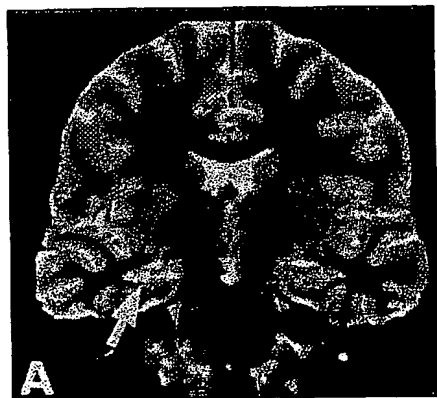


FIG. 2A

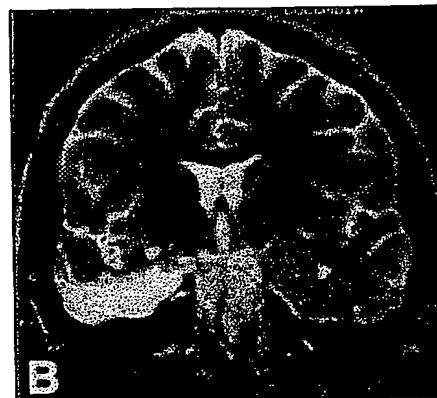


FIG. 2B

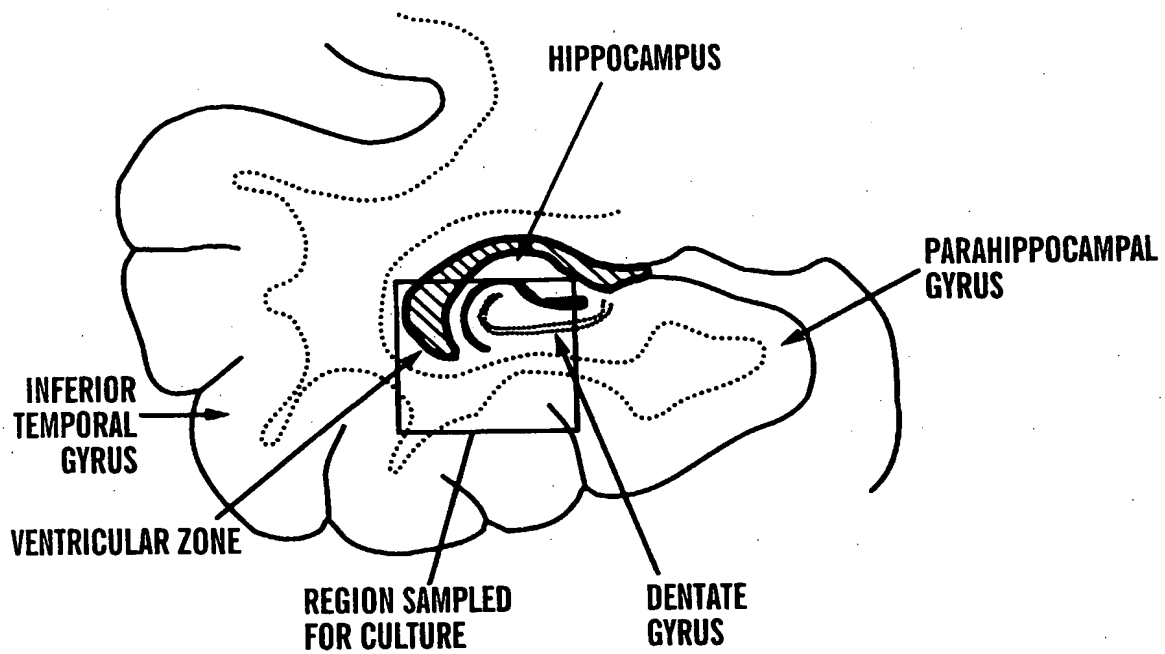


FIG. 2C

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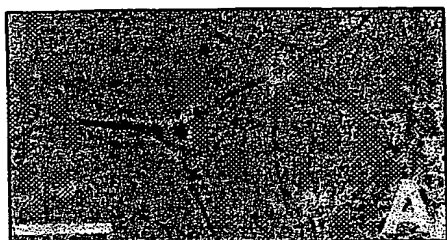


FIG. 3A

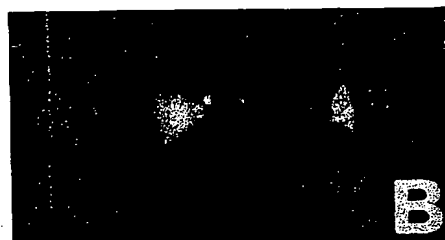


FIG. 3B

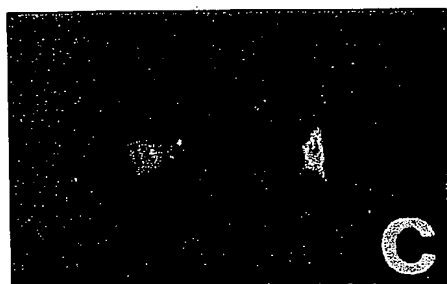


FIG. 3C



FIG. 3D

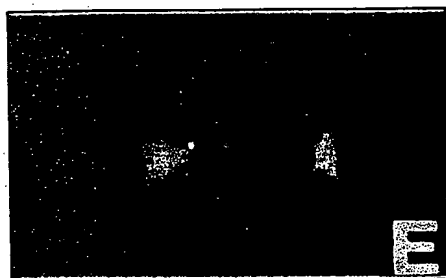


FIG. 3E

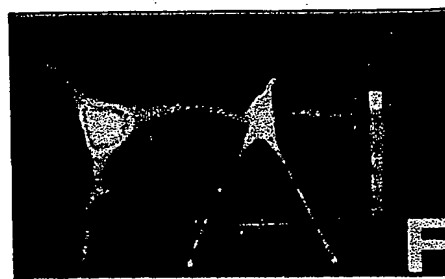


FIG. 3F

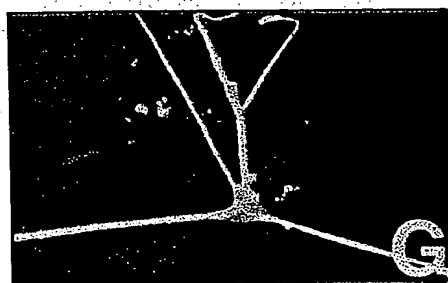


FIG. 3G

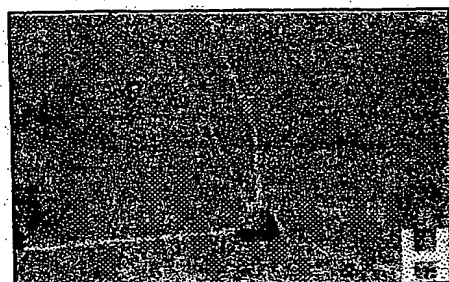


FIG. 3H

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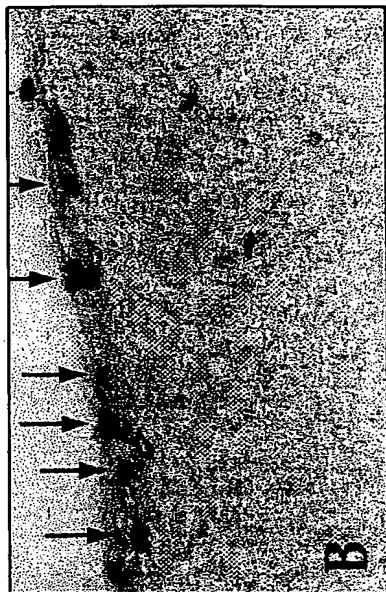


FIG. 4B

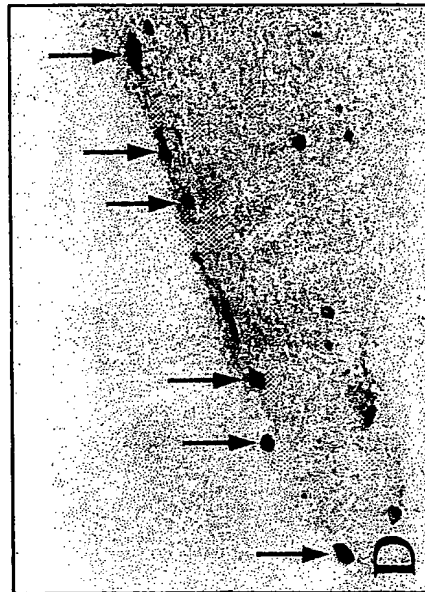


FIG. 4D

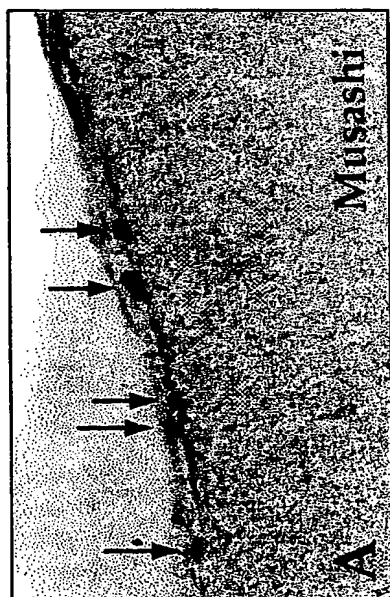


FIG. 4A

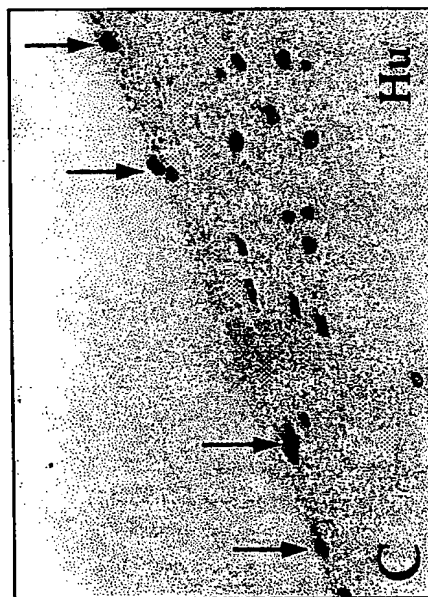


FIG. 4C

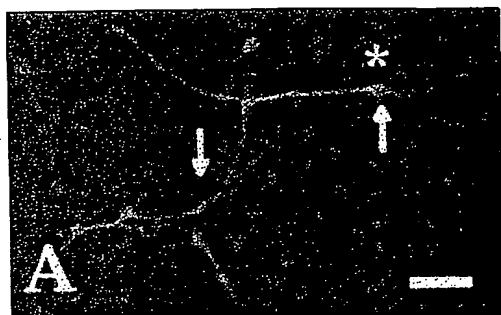


FIG. 5A

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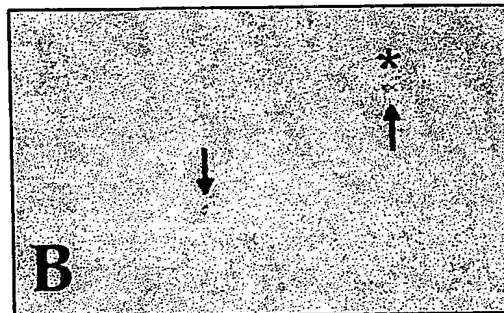


FIG. 5B



FIG. 5C

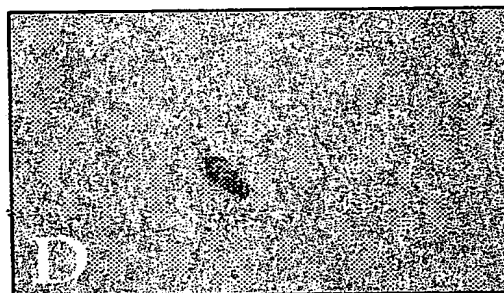


FIG. 5D

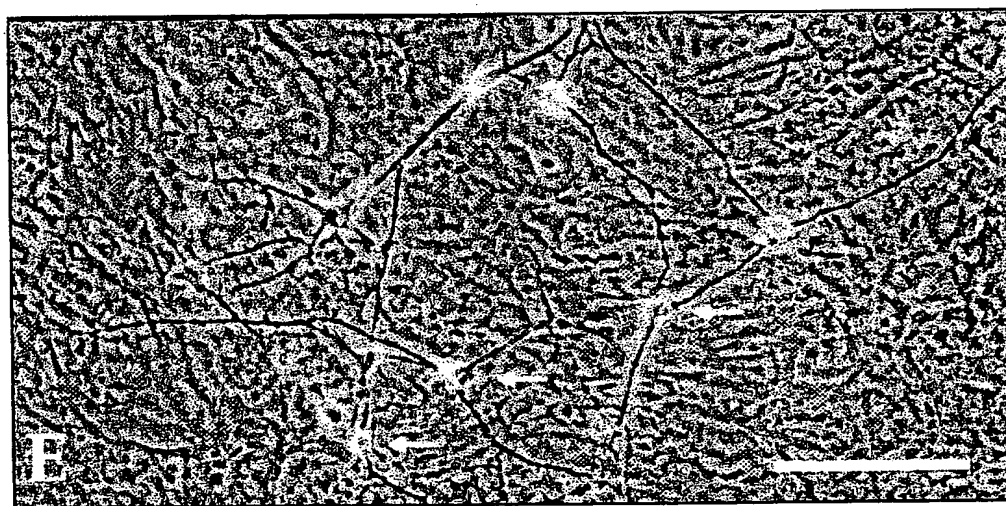


FIG. 5E

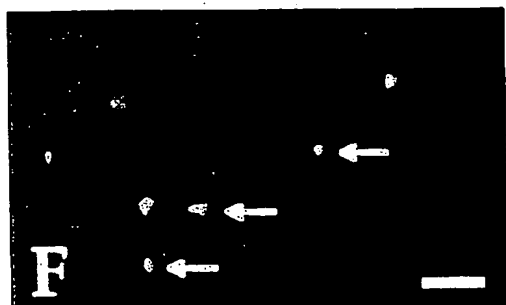


FIG. 5F

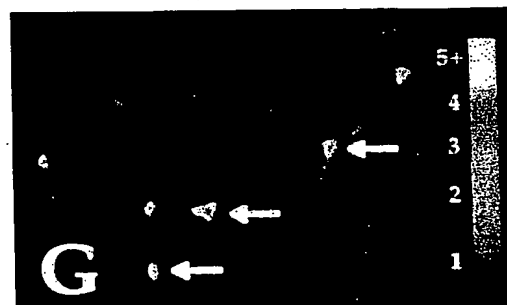
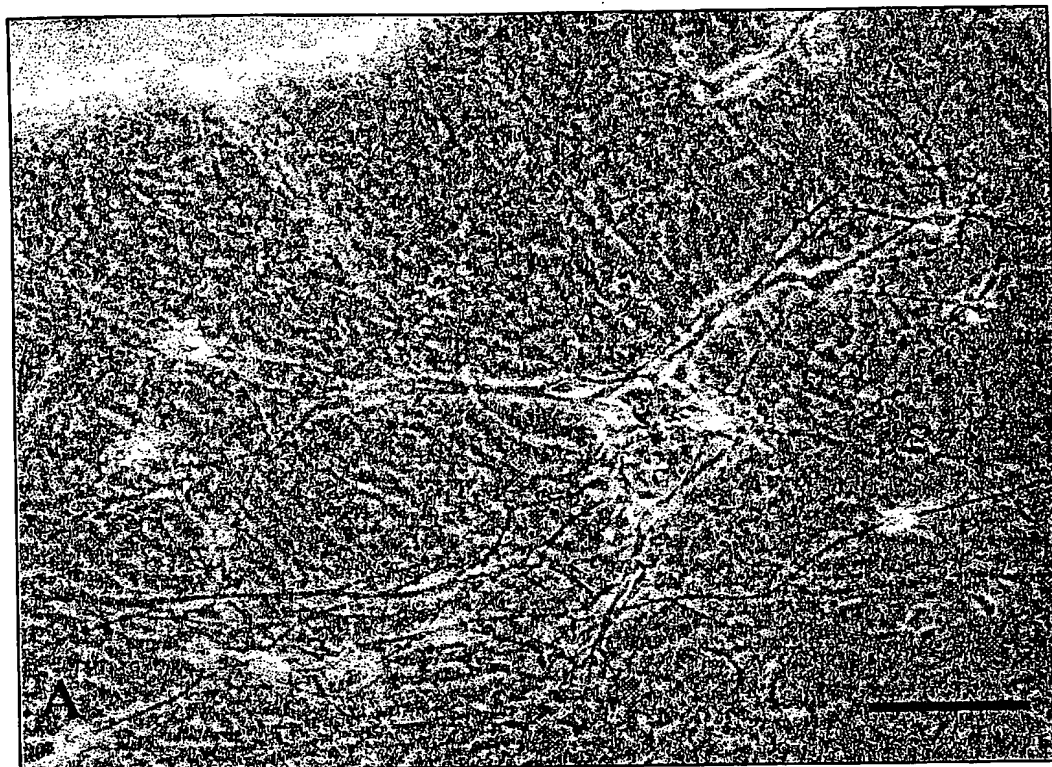
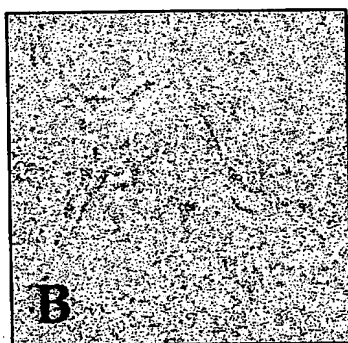
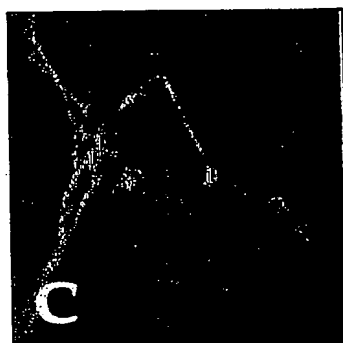
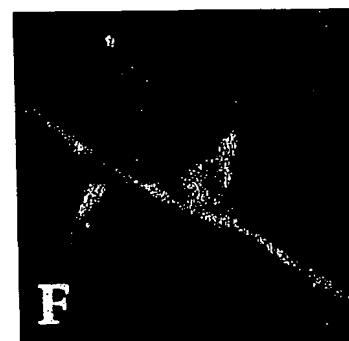
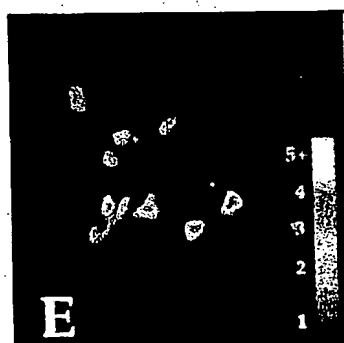
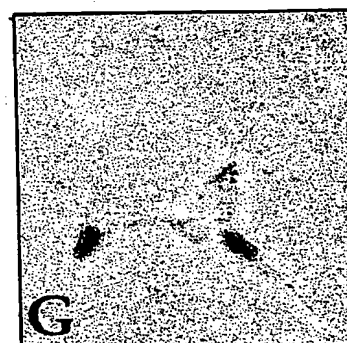


FIG. 5G

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**FIG. 6A****FIG. 6B****FIG. 6C****FIG. 6F****FIG. 6D****FIG. 6E****FIG. 6G**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06227

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 01275 A (WEISS SAMUEL ;REYNOLDS BRENT A (CA)) 21 January 1993 (1993-01-21) the whole document	1,3, 7-12, 14-17, 19-24, 29,33,38
X	WO 94 02593 A (STEMPLE DEREK L ;ANDERSON DAVID J (US); CALIFORNIA INST OF TECHN ()) 3 February 1994 (1994-02-03) the whole document	1,3, 7-12, 14-24, 29,33, 38-41



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

30 July 1999

Date of mailing of the international search report

12/08/1999

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Panzica, G

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 07200 A (UNIV LELAND STANFORD JUNIOR ;BARRES BARBARA A (US)) 27 February 1997 (1997-02-27) the whole document ----	1,3, 7-12,14, 16,18, 29,38,40
X	WO 96 38576 A (SIGNAL PHARM INC) 5 December 1996 (1996-12-05)	1,3, 7-12, 14-24, 29,33, 38-40
Y	abstract examples table 2 claims ----	5,6, 25-28, 34-37
A	WANG S. ET AL.: "Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the Talphal tubulin promoter" NATURE BIOTECHNOLOGY, vol. 16, no. 2, February 1998 (1998-02), pages 196-201, XP002108528 cited in the application abstract ----	38-40
X,P	PINCUS D. ET AL.: "Fibroblast growth factor-2/brain-derived neurotropic factor-associated maturation of new neurons generated from adult human subependymal cells" ANNALS OF NEUROLOGY, vol. 43, no. 5, May 1998 (1998-05), pages 576-585, XP002108529 cited in the application the whole document ----	1-24
Y	GRINSPAN J.B., FRANCESCHINI B.: "Platelet-derived growth factor is a survival factor of PSA-NCAM+ oligodendrocyte pre-progenitor cells" JOURNAL OF NEUROSCIENCE RESEARCH, vol. 41, no. 4, 1995, pages 540-551, XP002108530 abstract ----	25-28
Y	KAHN A. ET AL.: "THERAPIE GENIQUE DES MALADIES NEUROLOGIQUES" C R SEANCES SOC BIOL FIL, vol. 190, no. 1, 1996, pages 9-11, XP002108531 FR abstract ----	34-37

INTERNATIONAL SEARCH REPORT

In tional Application No

PCT/US 99/06227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>BARAMI ET AL.: "Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain"</p> <p>JOURNAL OF NEUROBIOLOGY, vol. 28, 1995, pages 82-101, XP002108589 cited in the application abstract</p> <p>-----</p>	5,6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/06227

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 14-20 and 33-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Insofar as claims 21-28 may be intended as in relation to in vivo methods of treatment to be performed on a human/animal body, then objection arises under Art.17.2.a.1 PCT (subject matter not to be searched by the ISA, under Rule 39.1.IV PCT)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/06227

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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